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(54) Title: METHODS FOR HIGH THROUGHPUT AND QUANTITATIVE PROTEOME ANALYSIS
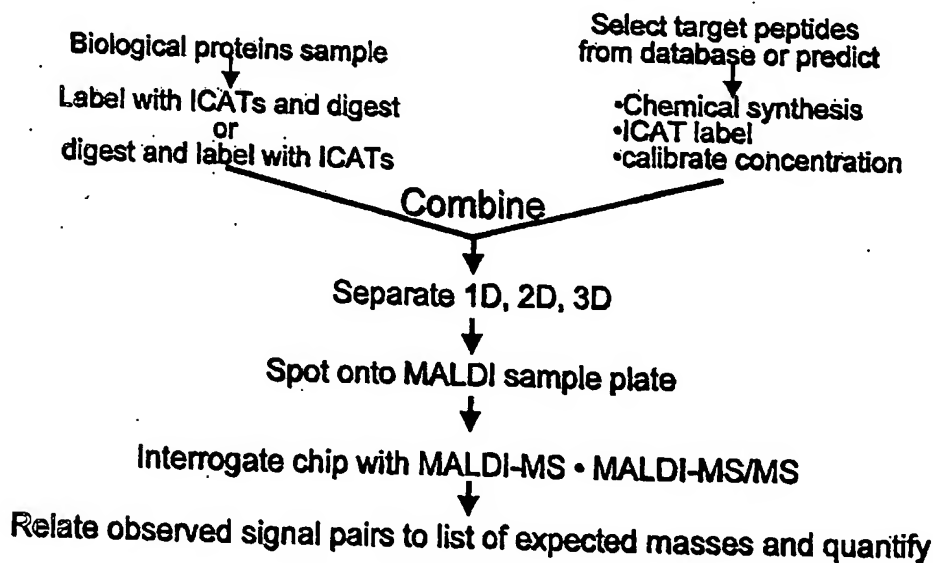
(57) Abstract: The invention provides methods for identifying and quantifying polypeptides in a sample. The methods include the steps of labeling peptides in a polypeptide sample with an isotope tag; adding a plurality of peptide standards to the polypeptide sample, wherein the peptide standards are labeled with an isotopically distinct version of the isotope tag; resolving the labeled sample and standard peptides into a plurality of fractions, analyzing the resolved fractions using mass spectrometry, identifying an isotope-tagged sample peptide in an analyzed fraction; and determining the amount of the identified isotope-tagged sample peptide in the analyzed fraction by comparison to the amount of isotope tagged standard peptide in the same fraction.

METHODS FOR HIGH THROUGHPUT AND QUANTITATIVE PROTEOME ANALYSIS

10   ## BACKGROUND OF THE INVENTION

The present invention relates generally to proteomics and more specifically to quantitative proteomics analysis.

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Complete genomic sequences and large partial (EST) sequence databases can potentially allow the identification of every gene in a species. However, the sequences alone do not explain the mechanism of
20   biological and clinical processes because neither the amount nor the activity of the protein products can be easily predicted from the gene sequence. From genomic analysis or the analysis of the expressed mRNA transcripts, neither the quantity nor the structure,
25   activity and state of modification of the translated protein products can be predicted. Furthermore, the gene sequence alone cannot be used to reliably predict whether and how a gene will be spliced and how and at what position a protein is modified.

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In order to assess the physiological state of
a cell or organism using proteomics, it is important to
understand the nature of protein modifications and the
quantities of expressed proteins. As biological
5 systems are dynamic, such technologies need to be
quantitative.   Such an analysis requires methods for
the determination of the absolute quantity of each
protein in a biological or clinical sample and for the
determination of the precise composition of the
10 proteins. This includes the determination of splice
forms and modifications.

A number of approaches have been used to
address the needs of proteomics analysis. For example,
15 the combination of two-dimensional gel electrophoresis
(2DE) and protein identification by mass spectrometry
(MS) or tandem MS (MS/MS) constitute such a method.
However, a limitation to this approach is that 2DE-MS
analysis does not provide a true representation of. the
20 proteins in a biological sample because specific
classes of proteins are known to be absent or under
represented in 2D gel patterns. These include very
acidic or basic proteins, excessively large or small
proteins, membrane proteins and other proteins of poor
25 solubility in aqueous solvents, and low abundance
proteins.

Other methods for proteome analysis include
quantitative mass spectrometry based on
30 multidimensional peptide separation and isotope coded
affinity tagging of proteins. This method allows
relative quantitation, that is, the determination of
the abundance ratio of each protein in two samples but
does not allow determination of the absolute quantity
35 of the proteins in a sample. Also, chip technology
using arrays of reagents with known specificity for

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target proteins such as antibody arrays or arrays of
aptamers can be used for proteomics analysis. However,
the use of such arrays can be limited by the need to
selectively capture representative proteins or preserve
5   the three dimensional structure of the proteins
depending on the particular use of the chip.

Mass spectrometry (MS) based methods for
proteomics have in common that the currency of protein
10   identification and quantification is a peptide
generated by the sequence specific fragmentation of a
protein. Therefore, proteins need to be enzymatically
or chemically fragmented prior to mass spectrometric
analysis. Furthermore, the MS based proteomic methods,
15   alone or in conjunction with other methods, have in
common that throughput is limited by the need to
sequence each peptide in each sample in each experiment
to determine the sequence identity of the protein
analyzed. A protein generally generates a large number
20   of peptides and hence a large number of peptides has to
be sequenced per experiment. The yeast proteome is
estimated to contain approximately 6000 open reading
frames (ORF's), which would generate approximately
300,000 to 400,000 tryptic peptides, depending on how
25   specifically the enzyme works to cleave the yeast
proteins. Thus, a huge number of peptides would need
to be analyzed for determination of the physiological
state in a sample; even if only a subset of all
possible genes is expressed in a cell at a given state.
30

Thus, there exists a need for methods of high
throughput and quantitative proteome analysis. The
present invention satisfies this need and provides
related advantages as well.

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SUMMARY OF THE INVENTION

The invention provides methods for
5   identifying and quantifying polypeptides in a sample.
The methods include the steps of labeling peptides in a
polypeptide sample with an isotope tag; adding a
plurality of peptide standards to the polypeptide
sample, wherein the peptide standards are labeled with
10   an isotopically distinct version of the isotope tag;
resolving the labeled sample and standard peptides into
a plurality of fractions; analyzing the resolved
fractions using mass spectrometry; identifying an
isotope-tagged sample peptide in an analyzed fraction;
15   and determining the amount of the identified isotope-
tagged sample peptide in the analyzed fraction by
comparison to the amount of isotope tagged standard
peptide in the same fraction.

20   **BRIEF DESCRIPTION OF THE DRAWINGS**

Figure 1 shows a flow chart for the
generation of ordered peptide arrays for quantitative
25   protein profiling using calibrated synthetic external
standard peptides. Sample and standard peptides are
isotopically labeled, combined, and analyzed by mass
spectrometry (MS).

30   Figure 2 shows a schematic diagram of an
ordered peptide array and read out by matrix-assisted

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laser desorption/ionization (MALDI-MS). The sticks in
the scheme indicate peptide signals that are detected
by the MALDI-TOF mass spectrometer. It is apparent
that some signals are detected as pairs of a mass
5   difference that corresponds to the mass difference
encoded in the isotope tag. These are indicated in
yellow. The other signals appear as singlets and are
not further considered for analysis because their mass
does not correspond to the mass of an externally added
10   standard peptide.

     Figure 3 shows a particular embodiment of the
separation protocols, isotopic labeling, and MS
analysis used for quantitative protein profiling.
15

      .Figure 4 shows a schematic representation of
a method for generating ordered peptide arrays using
capillary reverse-phase chromatography and spotting of
the eluting peptides on a MALDI-MS sample plate.
20

     Figure 5 shows a schematic representation of
the method of protein profiling. A desired set of
proteins to be profiled is selected, combined with
sample peptides, and analyzed by MS.
25

     Figure 6 shows a schematic representation of
the mass spectrometry analysis for protein profiling.
Proteins are resolved into fractions, which are
deposited on a MS plate, and analyzed by MALDI-MS/MS to
30   generate a protein profile. Differential isotopically
labeled pairs of standard and sample peptides are
indicated by "*".

     Figure 7 shows a diagram of a format for
35   protein profiling. Figure 7A shows a schematic
representation of the fourplexed RP-μLC system. The

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system components can be segmented into four modules:
sample loading, solvent delivery, separation, and
fractionation. Figure 7B shows the flow-path and valve
configurations at the injection stage of the fourplexed
5 RP-µLC system. Figure 7C shows the flow-path and valve
configurations at the separation stage of the
fourplexed RP-µLC system.

Figure 8 shows elution profiles of ICAT^{TM}
10 reagent labeled bovine serum albumin (BSA) tryptic
peptides eluted from the four parallel columns.
Peptides were identified using a MALDI QqTOF mass
spectrometer.

15 Figure 9 shows quantitative peptide profiling
via MALDI-MS and MALDI-MS/MS. Protonated peptide
masses (M+H) of automatically determined, putative
ICAT^{TM} reagent labeled peptides derived from human
prostate cells are plotted against chromatographic
20 retention time. Circles indicate constitutively
represented peptides, while shaded squares indicate
peptides showing significant abundance changes.
Representative results from two identified,
differentially expressed peptides (SEQ ID NOS:1 and 2,
25 left and right) are shown.

DETAILED DESCRIPTION OF THE INVENTION

The invention provides methods to determine
30 the absolute quantity of proteins present in a
biological sample rapidly and in an automated manner.
The methods can be used to detect and quantify splicing
and other isoforms as well as specific modifications of
sample polypeptides. The methods are based on
35 generating an ordered array of differentially
isotopically tagged pairs of peptides, each pair

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representing a unique protein, a specific protein
isoform, or a specifically modified form of a protein.
One element of the peptide pairs is a synthetically
generated, external standard and the other element of
5   the pair is a peptide generated by enzymatic digestion
of the proteins in the sample mixture. The peptide
array is generated by separating the peptide mixtures
via a sequence of reproducible separation steps and
depositing the final peptide fractions on the sample
10   plate of a MALDI mass spectrometer. The peptide array
is then interrogated by a mass spectrometer to identify
the proteins present in a biological or clinical sample
and to determine the absolute abundance of each. The
position on the array, which reflects the separation
15. coordinates of the separation systems used, in
conjunction with the precise mass measurement of the
peptide, uniquely identifies each peptide and therefore
the protein it represents. The methods can be used to
build on the availability of whole genome sequences, of
20   software tools for the prediction of open reading
frames (ORF)'s, splice isoforms, modification sites and
also on the large amount of experimental data that are
being accumulated by large scale protein measuring
projects.
25

        The methods of the invention essentially
change a mass spectrometry based quantitative
proteomics experiment from a shotgun sequencing
approach, in which in every experiment each detected
30   peptide is sequenced by MS/MS, into a targeted
interrogation of the sample, in which the presence and
quantity of a predetermined set of peptides is
determined. The methods of the invention can include
the following steps: generation of isotope tagged,
35   calibrated peptide samples; preparation and isotope
tagging of protein sample; generation of the ordered

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peptide array; interrogation of the ordered array by
mass spectrometry or tandem mass spectrometry; and data.
analysis and display.

5   In one embodiment, the invention provides a
method for identifying and/or quantifying polypeptides
in a sample. The method can include the steps of
labeling polypeptides in a·polypeptide sample with an
isotope tag; adding a plurality of peptide standards to
10   the polypeptide sample, wherein the peptide standards
are labeled with an isotopically distinct version of
the isotope tag; resolving the labeled sample and
standard peptides into a plurality of fractions;
analyzing the resolved fractions using mass
15   spectrometry; identifying an isotope-tagged sample
peptide in an analyzed fraction; and determining the
amount of· the identified isotope-tagged sample peptide
in the analyzed fraction by comparison to the amount of
isotope tagged standard peptide in the same fraction.
20

          An exemplary embodiment of the invention is
shown in Figure 1. A set of target peptides is
selected from a database or predicted from known
sequences. A set of target peptides is synthesized
25   that would correspond to a predetermined fragmentation
of parent polypeptides, for example, digestion with a
protease or chemical cleavage, and.labeled with an
isotope tag, here illustrated as an ICAT™ label. The
standard ·peptides are calibrated so that absolute
30   amounts are known and added for comparison and
quantification. A sample of interest, such as a
biological sample, is also labeled with the same
isotope tag as used for the standard peptides except
differing in the isotopic label. Polypeptides in the
35   sample are digested with a protease or chemically
cleaved in the same manner as for the selected target

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peptides. The sample and standard peptides are combined and resolved in parallel using one or more fractionation techniques, for example, 1, 2 or 3 dimensional modes of separation. The fractions are

5   spotted on a MALDI sample plate. The arrayed fractions are interrogated with MALDI-MS and/or MALDI-MS/MS. Paired signals corresponding to differentially labeled sample and standard peptides are observed and related to a list of expected masses based on the particular

10 standard peptides included. The addition of known amounts of standard peptides also allows for quantification of the corresponding sample peptides.

The methods of the invention are useful for
15 proteome analysis and can be used to identify and quantify multiple polypeptides in a complex sample. The methods can be used, for example, for blood serum profiling, clinical applications, analysis of the physiological state of a biological sample, splice
20 isoform mapping and profiling, and mapping and profiling of post-translational modifications.

As used herein, the term "polypeptide" refers to a peptide or polypeptide of two or more amino acids.
25 A polypeptide can also be modified by naturally occurring modifications such as post-translational modifications, including phosphorylation, fatty acylation, prenylation, sulfation, hydroxylation, acetylation, addition of carbohydrate, addition of
30 prosthetic groups or cofactors, formation of disulfide bonds, proteolysis, assembly into macromolecular complexes, and the like.

As used herein, the term "sample" is intended
35 to mean any biological fluid, cell, tissue, organ or portion thereof, that includes one or more different.

10

molecules such as nucleic acids, polypeptides, or small
molecules. A sample can be a tissue section obtained
by biopsy, or cells that are placed in or adapted to
tissue culture. A sample can also be a biological

5   fluid specimen such as blood or plasma, cerebrospinal
fluid, urine, saliva, seminal plasma, pancreatic juice,
and the like. A sample can additionally be a cell
extract from any species, including prokaryotic and
eukaryotic cells as well as viruses. A tissue or

10 biological fluid specimen can be further fractionated,
if desired, to a fraction containing particular cell
types.

        As used herein, a "polypeptide sample" refers

15 to a sample containing two or more different
polypeptides. A polypeptide sample can include tens,
hundreds, or even thousands or more different
polypeptides. A polypeptide sample can also include
non-protein molecules so long as the sample contains

20 polypeptides. A polypeptide sample can be a whole cell
or tissue extract or can be a biological fluid.
Furthermore, a polypeptide sample can be fractionated
using well known methods, as disclosed herein, into
partially or substantially purified protein fractions.

25

        The use of biological fluids such as a body
fluid as a sample source is particularly useful in
methods of the invention. Biological fluid specimens
are generally readily accessible and available in

30 relatively large quantities for clinical analysis.
Biological fluids can be used to analyze diagnostic and
prognostic markers for various diseases. In addition
to ready accessibility, body fluid specimens do not
require any prior knowledge of the specific organ or

35 the specific site in an organ that might be affected by
disease. Because body fluids, in particular blood, are

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in contact with numerous body organs, body fluids "pick
up" molecular signatures indicating pathology due to
secretion or cell lysis associated with a pathological
condition.

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        The methods of the invention are based on the
identification of distinct peptides which are unique
for a polypeptide and can therefore be used to identify
the presence and quantity of the polypeptide in a
10 sample.
Peptides uniquely identifying a protein can be selected
experimentally or computationally. Experimentally such
peptides are selected from databases that contain all
the peptides from a species that have been previously
15 observed, for example, in tandem mass spectrometry
experiments. Computationally such peptides are
selected by translating the complete genomic sequence
or the sequence of all predicted genes and their
splice forms into the corresponding amino acid
20 sequences, by applying the rules for cleavage that are
predictable with each chemical or enzymatic protein
cleavage reagent to these amino acid sequence, and by
computing the sequence, mass and other properties for
each of the generated peptides. From this database of
25 predicted peptides, a suitable selection of peptides
that are unique for each target protein is then
selected.

        Once a set of peptides is selected, the
30 selected set of standard peptides can be made by
synthesizing a peptide unique for a polypeptide and
tagging the standard peptide in a manner that allows
identification and quantification of the same unique
peptide derived from a sample polypeptide. The tagging
35 is carried out so that differential isotope tags can be
separately added to the standard peptides and the

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sample peptides. The tagged standard and sample
peptides are co-purified and analyzed by mass
spectrometry so that the isotope tagged peptides can be
identified. Due to the fact that the standard peptide
5 and the peptide derived from the sample are chemically
identical but isotopically distinguished, they co-
purify in the separation methods used. The addition of
a known amount of the standard therefore allows direct
comparison and determination of the amount of the
10 corresponding sample peptide.

The sample proteins are labeled with a
chemically identical but isotopically different tagging
reagent to the one used to generate the standard
15 peptide mixtures. The tagged protein sample is
digested using the protease that was planned in the
design of the standard peptides.

As used herein an "isotope tag" refers to a
20 chemical moiety having suitable chemical properties for
incorporation of an isotope, allowing the generation of
differentially labeled reagents which can be used to
differentially tag a polypeptide in two samples. The
isotope tag also has an appropriate composition to
25 allow incorporation of a stable isotope at one or more
atoms. A particularly useful stable isotope pair is
hydrogen and deuterium, which can be readily
distinguished using mass spectrometry as light and
heavy forms, respectively. Any of a number of isotopic
30 atoms can be incorporated into the isotope tag so long
as the heavy and light forms can be distinguished using
mass spectrometry, for example, ^{13}C, ^{15}N, ^{17}O, ^{18}O or ^{34}S.
Exemplary isotope tags include the 4,7,10-trioxa-1,13-
tridecanediamine based linker and its related
35 deuterated form, 2,2',3,3',11,11',12,12'-octadeutero-
4,7,10-trioxa-1,13-tridecanediamine, described by Gygi

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et al. (<u>Nature Biotechnol.</u> 17:994-999 (1999). Other
exemplary isotope tags have also been described
previously (see WO 00/11208, which is incorporated
herein by reference). In contrast to these previously
5   described isotope tags related to an ICAT-type reagent,
it is not required that an affinity tag be included in
the reagent since the polypeptides are already
isolated. One skilled in the art can readily determine
any of a number of appropriate isotope tags useful in
10 methods of the invention.

Thus, an isotope tag can be an alkyl, akenyl,
alkynyl, alkoxy, aryl, and the like, and can be
optionally substituted, for example, with O, S, N, and
15 the like, and can contain an amine, carboxyl,
sulfhydryl, and the like (see WO 00/11208). These and
other derivatives can be made in the same manner as
that disclosed herein using methods well known to those
skilled in the art. One skilled in the art will
20 readily recognize that a number of suitable chemical
groups can be used as an isotope tag so long as the
isotope tag can be differentially isotopically labeled.
The stable isotope tag can also be introduced via a
solid-phase stable isotope tag transfer method, such as
25 the one described by Zhou et al., <u>Nature Biotechnol.</u>
20:512-515 (2002).

The peptide fragments are tagged with an
isotope tag to facilitate MS analysis. In order to tag
30 the peptide fragments, the isotope tag contains a
reactive group that can react with a chemical group on
the peptide portion of the peptide fragments. A
reactive group is reactive with and therefore can be
covalently coupled to a molecule in a sample such as a
35 polypeptide. Reactive groups are well known to those
skilled in the art (see, for example, Hermanson,

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Bioconjugate Techniques, pp. 297-364, Academic Press,
San Diego (1996); Glazer et al., Laboratory Techniques
in Biochemistry and Molecular Biology: Chemical
Modification of Proteins, Chapter 3, pp. 68-120,
5   Elsevier Biomedical Press, New York (1975); Pierce
Catalog (1994), Pierce, Rockford IL). Any of a variety
of reactive groups can be incorporated into an isotope
tag for use in methods of the invention so long as the
reactive group can be covalently coupled to a
10   polypeptide or other desired molecule in a sample. For
example, a polypeptide can be coupled via a sulfhydryl
reactive group, which can react with free sulfhydryls
of cysteine or reduced cystines in a polypeptide. An
exemplary sulfhydryl reactive group includes an
15   iodoacetamido group (see Gygi et al., supra, 1999).
Other examplary sulfhydryl reactive groups include
maleimides, alkyl and aryl halides, haloacetyls, α-
haloacyls, pyridyl disulfides, aziridines, acrylolyls,
arylating agents and thiomethylsulfones.
20

A reactive group can also react with amines
such as the α-amino group of a peptide or the ε-amino
group of the side chain of Lys, for example,
imidoesters, N-hydroxysuccinimidyl esters (NHS),
25   isothiocyanates, isocyanates, acyl azides, sulfonyl
chlorides, aldehydes, ketones, glyoxals, epoxides
(oxiranes), carbonates, arylating agents,
carbodiimides, anhydrides, and the like. A reactive
group can also react with carboxyl groups found in Asp
30   or Glu or the C-terminus of a peptide, for example,
diazoalkanes, diazoacetyls, carbonyldiimidazole,
carbodiimides, and the like. A reactive group that
reacts with a hydroxyl group includes, for example,
epoxides, oxiranes, carbonyldiimidazoles, N,N'-
35   disuccinimidyl carbonates, N-hydroxycuccinimidyl
chloroformates, and the like. A reactive group can

also react with amino acids such as histidine, for

example, α-haloacids and amides; tyrosine, for example;

nitration and iodination; arginine, for example,

butanedione, phenylglyoxal, and nitromalondialdehyde;

5   methionine, for example, iodoacetic acid and

iodoacetamide; and tryptophan, for example, 2-(2-

nitrophenylsulfenyl)-3-methyl-3-bromoindolenine (BNPS-

skatole), N-bromosuccinimide, formylation, and

sulfenylation (Glazer et al., *supra*, 1975). In

10 addition, a reactive group can also react with a

phosphate group for selective labeling of

phosphopeptides (Zhou et al., <u>Nat. Biotechnol.</u>, 19:375-

378 (2001)), or with other covalently modified

peptides, including lipopeptides, or any of the known

15 covalent polypeptide modifications. One skilled in the

art can readily determine conditions for modifying

sample molecules by using various reagents, incubation

conditions and time of incubation to obtain conditions

optimal for modification of molecule with an isotope

20 tag. The use of covalent-chemistry based isolation

methods is particularly useful due to the highly

specific nature of the binding of the polypeptides.

        The reactive groups described above can form

25 a covalent bond with the target sample molecule.

However, it is understood that an isotope tag can

contain a reactive group that can non-covalently

interact with a sample molecule so long as the

interaction has high specificity and affinity.

30

        A particularly useful method for labeling

sample polypeptides is the use of the isotope-coded

affinity tag (ICAT™) method (Gygi et al., <u>Nature</u>

<u>Biotechnol.</u> 17:994-999 (1999); WO 00/11208; each of

35 which is incorporated herein by reference). The

labeling procedures are essentially the same as

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developed for ICAT reagent protein labeling.   The
ICAT™ type reagent method uses an. affinity tag that can
be differentially labeled with an isotope that is   ·
readily distinguished using mass spectrometry. The
5   ICAT™ type affinity reagent consists of three elements,
an affinity tag, a linker and a reactive group.

One element of the ICAT™ type affinity
reagent is an affinity tag that allows isolation of
10   peptides coupled to the affinity reagent by binding to
a cognate binding partner of the affinity tag. A
particularly useful affinity tag is biotin, which binds
with high affinity to its cognate binding partner
avidin, or related molecules such as streptavidin, and
15   is therefore stable to further biochemical
manipulations. Any affinity tag can be used so long as
it provides sufficient binding affinity to its cognate
binding partner to allow isolation of peptides coupled
to the ICAT™ type affinity reagent. An affinity tag
20   can also be used to isolate a tagged peptide with
magnetic beads or other magnetic format suitable to
isolate a magnetic affinity tag. In the ICAT™ type
reagent method, or any other method of affinity tagging
a peptide, the use of covalent trapping can be used to
25   bind the tagged peptides to a solid support, if
desired.

A second element of the ICAT™ type affinity
reagent is a linker that can incorporate a stable
30   isotope. · The linker has a sufficient length to allow
the reactive group to bind to a specimen polypeptide
and the affinity tag to bind to its cognate binding
partner. The linker also has an appropriate
composition to allow incorporation of a stable isotope
35   at one or more atoms. A particularly useful stable
isotope pair is hydrogen and deuterium, which can be

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readily distinguished using mass spectrometry as light
and heavy forms, respectively. Any of a number of
isotopic atoms can be incorporated into the linker so
long as the heavy and light forms can be distinguished
5   using mass spectrometry. Exemplary linkers include the
4,7,10-trioxa-1,13-tridecanediamine based linker and
its related deuterated form, 2,2',3,3',11,11',12,12'-
octadeutero-4,7,10-trioxa-1,13-tridecanediamine,
described by Gygi et al. (supra, 1999). One skilled in
10   the art can readily determine any of a number of
appropriate linkers useful in an ICAT™ type affinity
reagent that satisfy the above-described criteria, as
described above for the isotope tag.

15   The third element of the ICAT™ type affinity
reagent is a reactive group, which can be covalently
coupled to a polypeptide in a specimen. Various
reactive groups have been described above with respect
to the isotope tag and can similarly be incorporated
20   into an ICAT-type reagent.

           The ICAT™ method or other similar methods can
be applied to the analysis of the peptide fragments.
The method generally involves the steps of automated
25   tandem mass spectrometry and sequence database
searching for peptide/protein identification; stable
isotope tagging for quantification by mass spectrometry
based on stable isotope dilution theory; and the use of
specific chemical reactions for the selective isolation
30   of specific peptides. For example, the previously
described ICAT™ reagent contained a sulfhydryl reactive
group, and therefore an ICAT™-type reagent can be used
to label cysteine-containing peptide fragments released
from the solid support. Other reactive groups, as
35   described above, can also be used.

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In addition to using isotope tags, other
types of tags can be used as long as the tags allow
differential labeling of calibrated standard peptides
and sample-derived peptides. For example, fluorescent
5 dyes that have the same mobility in the various
fractionation steps but different spectral properties
can be used. Similarly, other types of detectable
tags, such as chromophores or radioisotopes, can be
used. One skilled in the art can readily determine
10 appropriate detectable labels to differentially label
calibrated peptides and sample peptides so long as the
fractionation steps used in methods of the invention
provide sufficient separation space to separate the
target peptides.
15

If desired, sample molecules can be modified,
either before or after a fractionation step. For
example, the methods of the invention are particularly
useful for mass spectrometry (MS) analysis. In the
20 case of MS analysis of polypeptides, it is often useful
to cleave the polypeptide into smaller fragments, for
example, by proteolysis. Thus, a polypeptide molecule
can be enzymatically cleaved with one or more proteases
into peptide fragments. Exemplary proteases useful for
25 cleaving polypeptides include trypsin, chymotrypsin,
pepsin, papain, *Staphylococcus aureus* (V8) protease,
Submaxillaris protease, bromelain, thermolysin, and the
like. Polypeptides can also be cleaved chemically, for
example, using CNBr, acid or other chemical reagents.
30

For polypeptide fragmentation, the
polypeptides in the sample mixture, or the polypeptides
contained in each fraction if optional sample
fractionation is employed, can be subjected to specific
35 cleavage, for example, by trypsin. The use of sequence
specific cleavage can be particularly useful because

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the termini of peptides cleaved by a sequence specific
method can act as a constraint. However, it is
understood that the cleavage method used to generate
fragments need not be sequence specific, if desired.

5

        Furthermore, for polypeptide tagging, the
polypeptides in the sample can be denatured and
optionally reduced. Reducing the sample can be
particularly useful when the reactive group on the
10 tagging reagent is reactive with a thiol. Other useful
reactive groups include amino or carboxyl groups of
polypeptides or specific post-translational
modifications, including phosphate, carbohydrate or
lipid.

15

        For generation of standard peptides, a
collection of peptides are synthesized. These peptides
constitute the full space of proteome interrogation to
performed in a particular experiment. Each peptide in
20 the collection uniquely identifies a protein, a protein
isoform or a specifically modified form of a protein.
The sequence of the peptides is derived either from
empirical determinations, for example, MS/MS
experiments in which the peptide in question has been
25 observed, or by the application of computer programs
that select a set of peptides, including an optimal set
of peptides for a particular application. The computer
programs use the information contained in large protein
identification datasets, for example, which peptides
30 have been observed, genome sequences, with defined
ORFs, splice isoform databases, and the like, to select
one to a few peptides from each protein that uniquely
identifies the protein, a protein isoform or a specific
modification. Further important criteria for peptide
35 selection are the peptide mass, which needs to be
within the useful mass range of a mass spectrometer.

20

Also, desirable properties of the selected peptides
include solubility and physico-chemical features that
allow common peptide separation methods to separate the
peptides over a wide separation space. The peptides
5   can also contain one or several chemical groups that
can be targeted by isotope tagging reagents. In
general, peptides containing rare amino acids, such as
Cys, Met, and Trp, are selected so that the sample
complexity can be minimized by the selective isolation
10 of the peptides containing that amino acid.

        The peptide standards can be selected to
represent most or essentially all of the known
polypeptides in a sample. Alternatively, a subset of
15 polypeptides can be selected. For example, if a
particular set of polypeptides is desired to be
analyzed, then standard peptides are selected from this
set of polypeptides rather than from other polypeptides
known to be in the sample. This can be particularly
20 useful for diagnostic applications in which particular
diagnostic markers are to be analyzed rather than a
complete proteomics analysis of the sample. The amount
of standard peptides to be added can be adjusted, as
desired, to facilitate quantification, and each of the
25 peptide standards added need not be in the same
amounts.

        The peptide sequences, once selected, are
chemically synthesized by solid-phase stepwise
30 synthesis, stable isotope tagged and quantified.
Methods of synthesizing peptides are well known to
those skilled in the art (Merrifield, J. Am. Chem. Soc.
85:2149 (1964); Bodanszky, M., Principles of Peptide
Synthesis (Springer-Verlag, 1984); Houghten, Proc.
35 Natl. Acad. Sci., USA 82:5131 (1985)). For each
peptide, a calibrated sample stock solution is prepared

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and stored. Quantification of the calibrated stock solution can be carried out by amino acid composition analysis, can be based on UV absorbance measurement or other spectrometric methods, or by weighing the dried
5   peptide. The synthesized peptides can be isotopically tagged by reacting the standard peptides with an isotope tag, as described herein. Although isotope tagging is generally carried out in the same manner as the isotope tagging of the sample molecules, it is
10   understood that the isotope tagging of the peptide standards can be synthesized at the time of synthesis of the peptide standards so long as the resulting isotope tagged standard peptides differ from the corresponding isotope tagged sample peptides only by
15   the differential label of the isotope tag.

        Alternatively, the standard peptides can be generated by expression in a genetically engineered organism such as *Escherichia coli* or other
20   microorganisms. Each peptide can be expressed separately as a peptide product, as part of a larger polypeptide from which the peptide will be cut out by proteolysis, or in the form of concatenated peptides, which can be resolved into individual peptide species
25   by proteolysis or chemical cleavage at suitable sites. Once isolated, the peptides generated by genetic engineering and overexpression are isotopically labeled and used in the methods of the invention, as with the chemically synthesized peptides.
30

        For preparation of the protein samples, standard protocols are used to prepare and process the protein samples. Methods for preparing and processing protein samples are well known to those skilled in the
35   art (Scopes, <u>Protein Purification: Principles and Practice</u>, third edition, Springer-Verlag, New York

(1993)). If desired, the sample can be fractionated by
a number of known fractionation techniques.
Fractionation techniques can be applied at any of a
number of suitable points in the methods of the

5   invention. Thus, if desired, a substantially purified
sample fraction can be used. One skilled in the art
can readily determine appropriate steps for
fractionating sample molecules based on the needs of
the particular application of methods of the invention.

10

If desired, the sample can be fractionated by
a number of known fractionation techniques. Since such
fractionation methods separate molecules, such
techniques are used to resolve sample molecules. As

15 used herein, resolve, when used in reference to a
polypeptide or peptide, refers to the process of
separating a polypeptide or peptide from one or more
other polypeptides or peptides. Methods for resolving
sample molecules are well known to those skilled in the

20 art. Fractionation methods, which can be used to
resolve sample molecules, include but are not limited
to subcellular fractionation or chromatographic
techniques such as ion exchange, including strong and
weak anion and cation exchange resins, hydrophobic and

25 reverse phase, size exclusion, affinity, hydrophobic
charge-induction chromatography, dye-binding, and the
like (Ausubel et al., *supra*, 1999; Scopes, Protein
Purification: Principles and Practice, third edition,
Springer-Verlag, New York (1993); Burton and Harding,

30 J. Chromatogr. A 814:71-81 (1998)). Other
fractionation methods include, for example,
centrifugation, electrophoresis, the use of salts, and
the like (see Scopes, *supra*, 1993). One skilled in the
art will recognize that these and other fractionation

35 methods, which are well known to those skilled in the
art, can be used to resolve polypeptides or peptides.

23

Affinity chromatography can also be used including, for example, dye-binding resins such as Cibacron blue, substrate analogs, including analogs of

5   cofactors such as ATP, NAD, and the like, ligands, specific antibodies useful for immuno-affinity isolation, either polyclonal or monoclonal, and the like. Affinity chromatography can also be performed using DNA, lectins or other natural substances as an

10   affinity ligand. An exemplary affinity resin includes affinity resins that bind to specific affinity tags attached to the target protein, such as an affinity tag incorporated into an ICAT-type reagent. The resolution and capacity of particular chromatographic media are

15   known in the art and can be determined by those skilled in the art. The usefulness of a particular chromatographic separation for a particular application can similarly be assessed by those skilled in the art.

20   Those of skill in the art will be able to determine the appropriate chromatography conditions for a particular sample size or composition and will know how to obtain reproducible results for chromatographic separations under defined buffer, column dimension, and

25   flow rate conditions. The fractionation methods can optionally include the use of an internal standard for assessing the reproducibility of a particular chromatographic application or other fractionation method. Appropriate internal standards will vary

30   depending on the chromatographic medium or the fractionation method used. Those skilled in the art will be able to determine an internal standard applicable to a method of fractionation such as chromatography.

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24
Electrophoresis, including gel
electrophoresis or capillary electrophoresis, can also
be used to resolve sample molecules. As disclosed
herein, isoelectric focusing (IEF) is a particularly
5 useful method to resolve sample polypeptides. Other
types of electrophoresis can also be used, for example,
sodium dodecyl sulfate polyacrylamide gel
electrophoresis (SDS-PAGE) or acid-urea gel
electrophoresis. As discussed herein, sample molecules
10 can be processed, for example, by protease cleavage
into peptide fragments. Accordingly, when referring to
sample molecules, the sample molecules can be intact as
found in an original sample or can be processed, for
example, into smaller molecules such as peptides from a
15 polypeptide sample.

     For generation of the ordered peptide array,
a cocktail of the tagged, calibrated standard peptides
is added to a peptide mixture generated by the
20 digestion of the tagged protein sample. At this point,
the sample mixture consists of all the peptides
generated representing the digested protein sample and
an external, calibrated standard for each one of the
peptides that is be interrogated. The amount of tagged
25 standard peptides added is estimated from the expected
abundance of the protein in question. This abundance
can be estimated from the codon bias tables or from
previous experiments. The combined peptide mixture is
subjected to multidimensional separation. The specific
30 separation steps can include chromatography, for
example, reverse phase, ion exchange, and affinity
chromatography or other suitable chromatography steps,
as disclosed herein. Affinity chromatography can be
included to select tagged peptides in the case when a
35 rare amino acid has been targeted with an ICAT^{TM}
reagent or other types of affinity chromatography, as

disclosed herein; and gel electrophoresis, for example,
.IEF or other electrophoretic separations. The sample
is separated into a plurality of fractions using one or
more fractionation techniques, as disclosed herein.

The fractions containing separated peptides
can be spotted onto the sample plate of a MALDI mass
spectrometer in the format of an ordered array (Figure
2). The peptides are added with matrix mediating MALDI
ionization using standard protocols. The precise
address for each peptide has to be determined once or
can be computed from the known properties of the target
peptides. This can be done by sequencing of the
peptides deposited in each sample spot using a MALDI-
tandem mass spectrometer such as time-of-flight/time-
of-flight or quadruople time-of-flight MS (TOF-TOF or
qqTOF) or by the separation, deposition and MS analysis
of defined pools of peptides. On the peptide array,
each array element contains "expected" and "unexpected"
peptides. The "expected" peptides are identified as
paired signals of the differentially labeled standard
peptides and sample peptides (Figure 2).

A variety of mass spectrometry systems can be
employed in the methods of the invention for
identifying and/or quantifying a sample molecule such
as a polypeptide. Mass analyzers with high mass
accuracy, high sensitivity and high resolution include,
but are not limited to, ion trap, triple quadrupole,
and time-of-flight, quadrupole time-of-flight mass
spectrometers and Fourier transform ion cyclotron mass
analyzers (FT-ICR-MS). Mass spectrometers are
typically equipped with matrix-assisted laser
desorption (MALDI) or electrospray ionization (ESI) ion
sources, although other methods of peptide ionization
can also be used. In ion trap MS, analytes are ionized

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by electrospray ionization or MALDI and then put into an ion trap. Trapped ions can then be separately analyzed by MS upon selective release from the ion trap. Fragments can also be generated in the ion trap

5   and analyzed. Sample molecules such as polypeptides labeled with an ICAT^{TM} type reagent can be analyzed, for example, by single stage mass spectrometry with a MALDI-TOF or ESI-TOF system. Methods of mass spectrometry analysis are well known to those skilled

10   in the art (see, for example, Yates, J. Mass Spect. 33:1-19 (1998); Kinter and Sherman, Protein Sequencing and Identification Using Tandem Mass Spectrometry, John Wiley & Sons, New York (2000); Aebersold and Goodlett, Chem. Rev. 101:269-295 (2001); Aebersold and Mann,

15   Nature 422:198-207 (2003)).

      While mass spectrometers using MALDI ionization are particularly useful in methods of the invention, it is understood that mass spectrometers

20   equipped with ion sources of different types are also applicable in the methods of the invention. Specifically, mass spectrometers equipped with ESI ion sources are also suitable for methods of the invention. These include electrospray ionization time-of-flight

25   (ESI-TOF) mass spectrometers and ESI aTOF, ion trap, triple quadrupole and FT-MS mass spectrometers.

      Once the peptides are deposited as ordered arrays on the sample plate, they are analyzed by MS,

30   for example, in a MALDI-TOF mass spectrometer. The instrument is programmed to sequentially generate mass spectra from each sample spot. Data acquisition from each spot is continued with further laser shots until mass spectra of a specific quality to detect the

35   signals corresponding to peptide pairs have been generated. The mass spectra from each spot are written

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to a data-table and stored. Current mass spectrometers
have a laser frequency of 20-30 Hz. It is expected
that the laser frequency will increase to 200Hz and
later to minimally 1000 Hz. It is also estimated that
5   for the interrogation of each sample spot, on the order
of 100 laser shots are required. Therefore at 200 Hz
laser frequency, the interrogation of a 96 spot sample
plate will take about one minute, indicating that the
methods can be applied for rapid and high throughput
10 sample analysis. It is understood that, based on the
needs of a particular sample analysis, one skilled in
the art can use any desired configuration of fraction
distribution on the array, including number of
fractions on a particular array, and number of
15 replicates, if desired. Furthermore, one skilled in
the art can modify the configuration based on the type
of MS analysis to be used, the number of fractions to
be analyzed, the speed of data acquisition, and the
desired accuracy of the data to be collected.
20

By distributing the resolved peptide
fractions onto a mass spectrometry sample plate, an
array is generated that can be used to assign the
identification of a peptide to a known location. Once
25 an array has been generated and defined and the
locations of identified peptides stored in a database,
a similar sample can be run under substantially similar
conditions and the location on the array used to
identify the peptide without the need for sequencing
30 the resolved peptides. Thus, subsequent analysis of a
similar sample can be performed more efficiently.
Rather than performing the more detailed analysis
required for sequencing, the MS analysis can be focused
on quantitative analysis as well as identification of
35 the peptides based on location on the array in
combination with other characteristics determined by

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the MS analysis or provided by the position of the
peptide on the array.

        The distribution of resolved peptide.
5 fractions onto a MS sample plate serves to facilitate
MS analysis, and further functions to convert the
resolution of the fractions into coordinates on an
array. As discussed above, once an array has been
generated and the identity of particular peptides is
10 correlated to a particular coordinate on the array, the
identification of a subsequently analyzed sample
peptide can be determined more efficiently based on its
position on a similarly generated array. Thus, the
distribution of fractions on an array and correlation
15 of coordinates on the array with the identity of a
peptide can be used to increase the efficiency of the
analysis of similarly processed samples. Although the
use of an array is particularly useful for such
analysis, it is understood that the distribution on an
20 array is not required to practice methods of the
invention. For example, the samples can be analyzed
using ESI-MS or ESI-MS/MS, without the need for
distribution of fractions as an array on a MS sample
plate. In such a case, information on the order of
25 elution and the retention times of the resolved
peptides is retained, for example, in a database.

        Data analysis can be carried out using the
following steps. First, in the mass spectrum of each
30 sample spot, the ion signals are detected and de-
isotoped. De-isotoping means collapsing all the
signals of the same peptide that reflect the natural
isotopic distribution (essentially the approximately
1% ^{13}C component of natural substances) and that are
35 resolved in high resolution mass spectrometers such as
the MALDI-MS system into a single peak. Second, pairs

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of peptide signals differing in mass by the number of
stable isotope atoms introduced as part of the isotope
tag in the sample versus standard peptides are
detected.

5

.Third, the signal for a specific ion pair is
followed over 3 consecutive sample spots and summed up
so as to minimize the impact of chromatographic isotope
effects on quantification. Thus, if a particular

10   peptide and its istopically labeled standard do not
reside in a single fraction, its presence in
neighboring fractions can be accounted for and added
together for the quantitative analysis. Fourth, the
detected peptide is identified by comparing its precise

15   mass and the separation coordinates reflected in the
array position with the entries in a database that
contains the precise mass and the separation
coordinates of all the standard peptides added to the
sample mixture and processed under substantially

20   similar conditions.

Fifth, from the combined signal intensities
for a peptide pair, the ratio of abundance is
calculated. Based on the known quantity of the

25   external standard peptide, the absolute amount of the
protein initially present in the sample is deduced.
This operation is repeated for each sample spot and the
data are compiled in a data-table for the experiment.
Thus, the methods of the invention can be used to

30   determine relative quantities of polypeptides present
in the original sample as well as absolute quantities
by comparison to the standard peptides.

An exemplary embodiment is shown in Figure 3.

35   In the illustrated embodiment, protein samples are
labeled with an isotope tag, the ICAT™ reagent. The

30

proteins are trypsinized and resolved by isolectric focusing (IEF) in immobilized pH gradient-IEF. Proteins are extracted from the gel and bound to avidin chromatography through the biotin affinity tag on the

5 ICAT™ reagent. Affinity isolated peptides are resolved by capillary liquid chromatography, and resolved fractions are spotted on a MALDI plate. The array of peptides is analyzed by MALDI-MS and/or MALDI-MS/MS. Standard peptides are added to the sample

10 peptides and resolved together for peptide identification and quantification.

Another embodiment of the invention is shown in Figure 4, which illustrates multiplex reverse phase

15 micro liquid chromatography. An ICAT™-labeled mixture of standard and sample peptides are resolved using strong cation exchange chromatography (SCX). The resolved fractions are affinity purified using the biotin tag of the ICAT™ reagent. The affinity

20 purified fractions are further resolved using reverse phase micro liquid chromatography. The resolved fractions are spotted on a MALDI plate and analyzed by MS. This illustrates that multiple chromatography steps can be used to resolve peptides for MS analysis.

25 The number of desired chromatography steps or other types of fractionation steps can be readily determined by one skilled in the art based on the needs of a particular application with respect to the need to resolve peptides sufficient for identification and/or

30 quantification.

When comparing experimental data with that of a database, it is understood that the experimental data is obtained from substantially the same sample type as

35 well as under substantially the same conditions. For example, if a serum sample is to be tested, the

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database or data-table for comparison of the sample
should also be made with a serum sample.
Alternatively, all the peptides from a species for
which genomic sequence information is known can be
5   calculated and stored in a single database, which can
be annotated with known information indicating the
tissue expression pattern, the subcellular location, or
other known characteristics of the protein. Thus, the
peptide standards that are used to generate the
10 original array and from which information is stored in
a data-table are added to a sample substantially
similar to the test sample. Once a standard set of
peptides has been resolved into identifiable fractions,
the same identifiable fractions will result when the
15 standard set of peptides is added to a test sample run
under substantially similar conditions. One skilled in
the art will know or can readily determine
substantially similar conditions suitable for
reproducible resolution of peptide fractions.
20

        The methods of the invention can be used in a
variety of applications. For example, the methods of
the invention can be used for profiling blood serum.
The ability to analyze readily accessible specimens
25 such as blood serum is particularly useful for clinical
applications. Thus, the methods are also applicable to
basic biology and clinical analysis.

        The methods of the invention can also be
30 applied to the analysis of splice isoform mapping and
profiling. Thus, differential splicing resulting in
protein splice isoforms can be readily tested at the
protein expression level. In the case of splice
variants, peptide standards can be selected to assess a
35 common portion as well as a portion of the sequence in
which the splice isoforms differ, if desired. Thus,

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the invention provides a method for the quantitative
profiling of splice and other protein isoforms. The
invention also provides a method for the determination
of the absolute quantities of splice and other protein
5   isoforms.

The methods of the invention are also
applicable to the mapping and profiling of post-
translational modifications. Thus, the invention
10   provides a method for the quantitative profiling of
post-translational modifications.

For profiling of polypeptides having post-
translational modifications, a modified peptide having
15   a known post-translational modification is chemically
synthesized and used in the methods of the invention,
as described above. Methods for the synthesis of
phosphorylated peptides are well known to those skilled
in the art, and other types of modifications readily
20   can be synthesized by those skilled in the art (Gerber
et al., Proc. Natl. Acad. Sci. USA 100:6940-6945
(2003)). For mapping post-translational
modifications, MS analysis can be used to identify
modified peptides and the corresponding post-
25   translational modifications.

The methods of the invention are advantageous
for a variety of applications. For example, the
methods allow the determination of absolute quantities
30   of sample polypeptides by the inclusion of and
comparison to known absolute amounts of standard
peptides. Thus, the invention provides a method for
quantitative proteome profiling, and can include the
analysis of each of the standard peptides added to the
35   test sample, if desired. It is understood, however,
that the analysis and/or quantification of each of the

added standard peptides need not be performed. The
invention also provides a method for quantitative
proteome profiling in which the absolute amount of
sample polypeptide is determined. In the case of post-
translational modification or isoform profiling, the
stoichiometry of the different isoforms and/or
modifications can be determined. Figure 5 illustrates
an exemplary embodiment, where a list of proteins in a
desired organelle, complex, splice forms, or having
post-translational modifications, is selected. In this
illustration, an automated system is shown such that a
robotic system selects suitable peptides from a library
of calibrated standards for analysis of a particular
sample. Sample peptides are labeled with a
corresponding differential isotope tag and digested
according to the designed standard peptides. Sample
and standard peptides are combined, resolved using one
or more fractionation methods, and analyzed by MS. The
results of the MS analysis are used to identify and/or
quantify sample peptides corresponding to the added
standard peptides.

The methods of the invention can be applied
to look specifically at subproteomes, if desired.
Subproteomes refer to fractions of the proteome of a
cell or tissue that can be reproducibly isolated.
Subproteomes include the protein contents of
organelles, for example, mitochondria, chloroplasts,
peroxisomes, lysosomes, and the like; subcellular
fractions such as nuclear fraction, microsomal
fraction, plasma membrane fraction, cytoplasmic
fraction; specific protein complexes such as
splicosomes or ribosomes; specific classes of enzymes,
for example, kinases, phosphatases, serine hydrolases,
and the like; or proteins that are modified by a common
post-translational modification, for example,

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phosphorylated or glycosylated proteins, or other post-
translational modifications. Figure 5 illustrates an
exemplary embodiment, where a list of proteins in a
desired organelle, complex, splice forms,

5   modifications, is selected. In this illustration, an
automated system is shown such that a robotic system
selects suitable peptides from a library of calibrated
standards for analysis of a particular sample. The
peptides are resolved (separated), subjected to MS

10 analysis and analyzed. The illustrated automated
system is capable of analyzing about one 96-well plate
per minute.

      If desired, the sample can be processed so
15 that a subset of polypeptides in the original sample is
analyzed (see Example I). For example, it is possible
to isolate glycopolypeptides by specifically absorbing
oxidized glycopolypeptides to a hydrazide resin. If
desired, N-glycosylated peptides can be selectively

20 analyzed by using an N-glycosidase to release
glycopeptides bound to the hydrazide resin. Methods of
isolating phosphoproteins are also well known to those
skilled in the art and can be applied to isolate a
subset of polypeptides that are phosphorylated (Zhou et

25 al., 19:375-378 (2001)).

      Thus, the invention also provides methods for
quantitative profiling of protein modifications, which
can be determined in absolute amounts when known
30 absolute amounts of standard peptides are added to the
sample peptides. The invention thus provides a method
of identifying and quantifying phosphorylated
polypeptides by isolating phosphorylated polypeptides
using well known methods and applying the methods of
35 the invention to profile the phosphorylated
polypeptides.

35

The invention also provides a method for
quantifying polypeptides in a sample. The method can
include the steps of labeling peptides in a polypeptide
sample with an isotope tag; adding a known absolute

5   amount of a plurality of peptide standards to the
polypeptide sample, wherein the peptide standards are
labeled with an isotopically distinct version of the
isotope tag; resolving the labeled sample and standard
peptides into a plurality of fractions; analyzing the

10 resolved fractions using mass spectrometry; identifying
an isotope-tagged sample peptide in an analyzed
fraction; and determining the amount of the identified
isotope-tagged sample peptide in the analyzed fraction
by comparison to the amount of isotope tagged standard

15 peptide in the same fraction.

The invention additionally provides a method
for identifying and/or quantifying splice isoforms of
polypeptides in a sample. The method can include the

20 steps of labeling peptides in a polypeptide sample with
an isotope tag; adding a plurality of peptide standards
to the polypeptide sample, wherein the peptide
standards are labeled with an isotopically distinct
version of the isotope tag and wherein the plurality of

25 peptide standards comprises at least one peptide
corresponding to a common amino acid sequence of a
splice isoform of a polypeptide and at least one
peptide corresponding to an amino acid sequence that
differs between two splice isoforms of the polypeptide;

30 resolving the labeled sample and standard peptides into
a plurality of fractions; analyzing the resolved
fractions using mass spectrometry; identifying an
isotope-tagged sample peptide in an analyzed fraction;
and determining the amount of the identified isotope-

35 tagged sample peptide in the analyzed fraction by

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comparison to the amount of isotope tagged standard peptide in the same fraction.

As discussed above, the methods of the
5   invention can be used to generate an array of known
peptides for a particular type of sample. The
information on the location of peptide standards on the
array can be stored in a database and referenced when
similar test samples are analyzed. Thus, the
10 information obtained and stored based on an original
set of peptide standards resolved under a given set of
conditions can be repeatedly accessed for comparison of
a similar test sample. Furthermore, different arrays
can be generated for different types of samples and the
15 information on peptide locations saved for future
analysis of similar types of test samples. Thus, the
methods of the invention can be used to develop
"arrays" that are specific for a number of
applications. Arrays can be generated to analyze
20 various types of samples, for example, blood serum,
lymphocytes, organelle or subcellular fractions such as
nuclear extracts, extracts of mitochondria,
chloroplasts, peroxisomes, lysosomes, membrane,
cytoplasmic fractions, and the like, as disclosed
25 herein.

The methods of the invention can be applied
to a number of species so long as a sufficient amount
of sequence information is available. The level of
30 sequence information sufficient to determine one or a
few signature peptides for a parent polypeptide can
readily be determined by one skilled in the art based
on the needs of the particular application. The
methods are particularly useful for organisms where
35 partial, nearly complete or essentially complete
genomic sequence is available. Thus, as the genomes of

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additional species are determined, the methods of the invention can be readily adapted for proteomics analysis of these species.

5   The methods of the invention are additionally advantageous because they obviate the need for antibodies, aptamers or other reagents that are specific for a particular protein for the analysis of polypeptide expression profiles. For some previously

10   described methods of proteome analysis, a specific antibody, aptamer or other type of specific reagent must be generated for each polypeptide to be analyzed. However, in the present invention, all that is needed is a synthetic peptide, which can be generated in a few

15   hours, and many peptides can be generated in parallel. Furthermore, the probes for sample analysis are easily standardized, quality controlled and distributed since the peptide standards can be accurately determined in absolute quantities and their chemical composition,

20   degree of purity and amino acid sequence are easily verified using well known methods. Because a set of easily synthesized peptide standards are used, the collection of peptide standards used to probe the test samples is easily updated if new genes or isoforms are

25   discovered.

        The methods of the invention allow the determination of relative and absolute quantities of polypeptides in a test sample. Polypeptides are

30   expressed at a wide range of amounts, with some very abundant polypeptides present in a sample along with low abundance polypeptides. The wide range of expression between individual polypeptides in a sample can cause difficulties in their analysis because the

35   low abundance proteins can be obscured and not easily detectable among the signals of the highly expressed

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proteins. The methods of the invention, however, allow an estimation of the approximate abundance of each peptide, reducing problems associated with the abundance range of polypeptides expressed in a sample.

The methods of the invention also provide sensitivity, speed, and high throughput for the analysis of complex polypeptide samples. By generating an ordered array, the location of specific peptides on the array can be determined once, with subsequent analysis of test samples not requiring the need to sequence peptides in order to identify them. In subsequent analysis of test samples, only the peptide mass has to be measured rather than determination of the sequence of the peptide, resulting in the method being very sensitive, in the subfemtomole range.

Furthermore, even if the peptides are sequenced in every experiment, that is, if the peptide sequence is not deduced by the position of the peptide on the array but by direct sequencing, for example, using a MALDI tandem mass spectrometer, the methods of the invention are still advantageous in terms of sample throughput. The peptides that are to be sequenced are characterized by their presence in the array as isotopic pairs of a precisely known mass difference. All the other peptides, that is, the peptides from proteins that are not being interrogated and other peptides from proteins for which one or a few peptides have been selected for analysis will appear as a singlet and can therefore be excluded from further analysis.

The invention additionally provides a set of polypeptides that uniquely identify a set of parent polypeptides. The invention also provides reagents and

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kits for identifying and quantifying polypeptides in a
sample. The kit can contain, for example, a collection
of peptide standards having an isotope tag. For
example, the kit can contain a set of calibrated

5   synthetic standard peptides of known relative or
absolute amounts. The kit can also include a set of
one or more isotope tags differentially labeled from
that of the standard peptides for coupling to sample
polypeptides, which are particularly useful for

10   quantitative analysis using mass spectrometry. The kit
can also contain one or more reagents for purification
of sample peptides, for example, chromatography
columns, electrophoresis gels, and the like, as well as
a protease(s) or other cleavage reagent corresponding

15   to the cleavage method used to derive the peptide
standards. The contents of the kit of the invention,
for example, any standard peptides or labeling
reagents, are contained in suitable packaging material,
and, if desired, a sterile, contaminant-free

20   environment. In addition, the packaging material can
contain instructions indicating how the materials
within the kit can be employed to label sample
molecules. The instructions for use typically include
a tangible expression describing the reagent

25   concentration or at least one assay method parameter,
such as the relative amounts of reagent and sample to
be admixed and how to adjust the amounts if needed for
quantification, maintenance time periods for
reagent/sample admixtures, temperature, buffer

30   conditions, and the like. The kits also can include
MALDI MS sample plates for the formation of the peptide
arrays, matrix molecules for MALDI MS and suitable
solvents for sample preparation.

35   The methods of the invention can be
facilitated by the use of combinations of hardware and

40

software suitable for analysis of methods of the
invention. Computer memory and data storage capacity as
well as appropriate algorithms can be used to
facilitate carrying out any or all of the following

5   steps of the invention or any other steps of the
invention: generation of a peptide database in which
the sequence and properties of the peptides added as
external standards are recorded; the step of detecting
the peptide pairs (sample and standard) and of

10 calculating the relative abundance of the peptide
pairs; the step of identifying the peptide by
correlating the separation coordinates and the precise
mass with the recorded database of sequence and other
properties of the peptides or by sequencing of the

15 peptides using, for example, a MALDI-tandem mass
spectrometer; the step of converting the individual
peptide datapoints into a quantitative protein profile
and of displaying that profile; and the step of
comparing multiple quantitative profiles with each

20 other for the detection of differences in the protein
expression profiles of different samples.

        An exemplary automated system is illustrated
in Figure 6. Figure 6 shows the generation of an

25 ordered peptide array using HPLC separation of
proteins. The ordered array(s) is analyzed by MS.
Pairs of differential isotopically labeled sample and
standard peptides are shown (*). The addition of a
known amount of standard peptides allows both

30 identification and quantification of the purified
peptides (Figure 6). Other peptides, which are not
paired with standard peptides, can be ignored, allowing
resources to be focused on the peptides of interest.

35   Figure 7 shows an embodiment of an automated
format for protein profiling. Figure 7A shows a

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schematic representation of the fourplexed reverse
phase micro fluid chromotography (RP-µLC) system. The
system components can be segmented into four modules:
sample loading, solvent delivery, separation, and
5   fractionation. Figure 7B shows the flow-path and valve
configurations at the injection stage of the fourplexed
RP-µLC system. Figure 7C shows the flow-path and valve
configurations at the separation stage of the
fourplexed RP-µLC system. It is understood that this
10   and a number of other arrangements of components is
suitable for use in methods of the invention.

     The invention provides methods of identifying
and/or quantifying polypeptides in a sample. It is
15   understood that methods of the invention can be carried
out in any suitable order so long as the desired
identification and quantification of sample peptides is
achieved. It is further understood that the methods
disclosed herein can be directed to identification
20   and/or quantification, as desired. The invention also
provides reports and methods of reporting the results
of methods of the invention for identifying and/or
quantifying polypeptides in a sample.

25   It is understood that modifications which do
not substantially affect the activity of the various
embodiments of this invention are also provided within
the definition of the invention provided herein.
Accordingly, the following examples are intended to
30   illustrate but not limit the present invention.

                    EXAMPLE I
          Quantitative Protein Profiling in Body Fluids

35   This example describes quantitative protein
profiling in a serum sample.

42

Human blood serum is subjected to a procedure
that selectively isolates a representative population
of peptides. Human blood serum is isolated using well
5 known methods, and polypeptides in the serum are
purified by well known methods (see, for example,
Scopes, *supra*, 1993). The serum proteins are denatured
in 6M urea and the disulfide bonds are reduced with
DTT, tributylphosphine, β-mercaptoethanol, tris-
10 carboxyethylphosphine (TCEP), or other common reducing
reagents. Proteins are then labeled with an ICAT
reagent (Gygi et al., *supra*, 1999) or similar isotope
tagging reagents following standard protocols. The
polypeptides are then digested with a protease such as
15 trypsin to generate peptide fragments.

The peptide fragments are tagged with a
suitable isotope tagging reagent. For example, the
peptide fragments can be tagged with an ICAT™-type
20 reagent. The reactive group can be reactive for a
subset of peptides, such as a sulfhydryl reactive group
which can couple to cysteine residues. If a large
number of the peptides are to be analyzed, for example,
in cases in which more complete sequence coverage is to
25 be achieved or if the selected peptide does not contain
a cysteine residue, a more general reactive group such
as an amino or carboxyl reactive group can be used
since the majority of cleaved peptides would contain a
free carboxyl and/or amino group. A collection of
30 peptide standards are synthesized and tagged with the
same tag used to tag the proteins except that the
peptide standards are differentially isotope tagged.
For example, the peptides can be labeled with an amino
group specific tagging reagent if glycopeptides are
35 bound to a hydrazide solid support (see below). The

labeled peptides are eluted, for example, using an N-glycanase, separated and sequenced.

The set of isotope-tagged peptide standards is added to the isotope-tagged sample peptides. The peptides are separated using one or more separation techniques and deposited on the sample plate in an array format. An exemplary separation protocol includes peptide separation by IEF followed by capillary reverse phase high performance liquid chromatography (RP-HPLC). Alternatively, the separation method can be multidimensional chromatography, for example, ion exchange, reverse phase, and the like. The separation method can also be extended single dimensional chromatography, where chromatographic separation is performed to optimize peptide separation with a single chromatography method, for example, with shallower slope of eluant.

A selected subset of the detected peptides are chemically synthesized and tagged with the light form of the reagent. Alternatively, the peptide standards can be tagged with the heavy form, so long as distinct isotopic forms are used between the standard and sample peptides. The subset is selected based on known sequences in the genome and/or previous empirical determination of the identity of polypeptides in the aserum sample. In some applications, the peptides are selected to achieve as complete coverage of the serum proteome as possible with easily separable, soluble and idiotypic peptides.

Typically, the peptide standard is added at the beginning of the process, generally after tryptic cleavage and before sample separation. In an initial experiment, before the selection of peptides for

synthesis is made, the digests of a serum sample prepared the same way are sequenced by tandem mass spectrometry.

5   Subsequent to the determination of the separation coordinates of the peptides selected for synthesis as standard peptides, similar serum protein samples to be profiled are processed in the same manner as the one used for the generation of the original

10 peptide list, except that the selected mixture of tagged standard peptides is added to the tagged serum sample after trypsinization. The combined mixture of sample and standard peptides labeled with differential isotopes are separated and spotted onto a MALDI-MS

15 sample plate. Each sample spot is interrogated by mass spectrometry.

      Alternatively, serum glycoproteins can be analyzed. For analysis of glycopolypeptides from serum

20 samples, 2.5 ml of human serum (200 mg total protein) such as human serum were changed to buffer containing 100 mM NaAc, 150 mM NaCl, pH 5.5 using a desalting column (Bio-Rad; Hercules CA). Sodium periodate solution at 15 mM was added to the samples. The cap

25 was secured and the tube is covered with foil. The sample was rotated end-over-end for 1 hour at room temperature. The sodium periodate was removed from the samples using a desalting column. A 50 μl aliquot of the sample was taken before coupling the sample. To

30 the sample was added 8 ml of coupling buffer equilibrated hydrazide resin (Bio-Rad). The sample and resin were capped securely and rotated end-over-end for 10-24 hours at room temperature. After the coupling reaction was complete, the resin was spun down at

35 1000xg for 10 min, and non-glycoproteins in the

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supernatant were removed. A 50 µl aliquot of the post
conjugation sample was taken.

5   For analysis of glycosylated peptides, non-
specific proteins bound to the resin were washed away
extensively by washing the resin 3 times with an equal
volume of 8M urea/0.4M NH₄HCO₃. The proteins on the
resin were denatured in 8M urea/0.4M NH₄HCO₃ at 55°C for
30 min, followed by 3 washes with the urea solution.
10   After the last wash and removal of the urea buffer, the
resin was diluted 4 times with water. Trypsin was
added at a concentration of 1 µg of trypsin/100 µg of
protein and digested at 37°C overnight. The trypsin
released peptides were removed by washing the resin
15   with an equal volume of 1.5 M NaCl for 3 times, 80%
MeCN/0.1% TFA for 3 times, 100% methanol for 3 times,
and 0.1 M NH₄HCO₃ for 6 times. The released non-
glycosylated peptides can be saved and optionally
labeled with an isotope tag for further analysis. The
20   bound glycopolypeptides can be labeled with an isotope
tag, essentially as described above, using an amino or
carboxyl reactive group on the isotope tagging reagent.
N-linked glycopeptides are released from the resin by
digestion with N-glycosidase at 37°C overnight. The
25   resin is spun and the supernatant saved. The resin is
washed twice with 80% MeCN/0.1% TFA and combined with
the supernatant. The resin is saved for O-linked
glycopeptide release later.

30   The peptides are dried in tubes, and one tube
is resuspended in 50 µl of 0.4% acetic acid. A 3 µl
aliquot of the sample is loaded on a capillary column
for µLC-MS/MS analysis. CID spectra are searched
against a database corresponding to the species of the
35   serum sample, for example, a human database, using
SEQUEST (Eng, J. et al., <u>J. Am. Soc. Mass. Spectrom.</u>

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5:976-989, (1994)) to identify the glycopeptides and
glycoproteins. The pairs of peptides differing by the
isotope tag are compared for quantification of the
corresponding sample peptides, as disclosed herein.

5

EXAMPLE II
Quantitative Profiling of Proteins Contained in Human
Mitochondria

10   This example describes profiling of proteins
in human mitochondria.

            Mitochondria, as an exemplary organelle, are
isolated using standard methods. Human cultured cells
15 grown in a suitable tissue culture medium are harvested
by centrifugation, washed free of serum by triplicate
washing in buffered saline solution and homogenized.
To isolate the mitochondria, the lysate is subjected to
a series of differential centrifugation steps. A
20 fraction highly enriched for mitochondria is isolated
from a 20,000 X g pellet by isopycnic centrifugation on
a discontinuous Nycodenz gradient. A protein extract
from the separated mitochondrial fraction is prepared
using standard methods (Scopes, *supra*, 1993). The
25 proteins are analyzed essentially as outlined in
Example I, except that the tagged standard peptides are
selected to represent all or the majority of known
human mitochondrial proteins. The peptide samples to
be synthesized are determined by sequence analysis of a
30 similarly prepared mitochondrial peptide sample.

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EXAMPLE III
Quantitative Profiling of Proteins Contained in Whole Cell Lysates

5   This examples describes protein profiling of whole cell lysates.

          Human cultured cells are grown and harvested essentially as described in Example II, except that
10   cultured cells are harvested by centrifugation and washing, and then the cells are lysed. Whole cell lysates are prepared using standard protocols (Scopes, *supra*, 1993). Lysis conditions to isolate most of the proteins is carried out by lysis of the cells in 1%
15   SDS.

          Protein profiling of the whole cell lysates is carried out essentially as described in Examples I and II except that the tagged standards are selected to
20   represent all or the majority of known human proteins or proteins known to be present in the particular tissue type corresponding to the cultured cells used for analysis.

25   EXAMPLE IV
Quantitative Profiling of Protein Splice Variants

          This example describes profiling of protein splice variants.
30
          A cell or tissue sample is prepared essentially as described in Examples II and III using well known methods (Scopes, *supra*, 1993). The peptides selected as external standards are chosen based on
35   known or empirically determined splice variants. The

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peptide standards are selected such that, for each
alternatively spliced protein, minimally one peptide is
used that is common to all splice variants and
minimally one peptide is used that is unique for each
5   splice variant tested. Protein profiling is carried
out essentially as described in Examples I-III.

EXAMPLE IV

Reproducible Fractionation of Peptides

10

        This example shows that parallel purification
is reproducible.

        Peptides were analyzed essentially as
15 described in Example I and disclosed herein. Briefly,
bovine serum albumin (BSA) was labeled with ICAT™
reagent essentially as described in Example I. Tryptic
digests were analyzed on parallel columns. Figure 8
shows the elution profiles of ICAT™ reagent labeled
20 BSA tryptic peptides eluted from the four parallel
columns. Peptides were identified using a MALDI QqTOF
mass spectrometer.

EXAMPLE V

25 Quantitative Peptide Profiling via MALDI-MS and MALDI-MS/MS

        This example shows quantitative peptide
profiling via MALDI-MS and MALDI-MS/MS.

30

        Samples derived from human prostate cells
were labeled with the ICAT™ reagent and analyzed
essentially as described in Example I and disclosed
herein. Figure 9 shows quantitative peptide profiling
35 via MALDI-MS and MALDI-MS/MS. Protonated peptide
masses (M+H) of automatically determined, putative

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ICAT™ reagent labeled peptides derived from human
prostate cells are plotted against chromatographic
retention time. Circles indicate constitutively
represented peptides, while shaded squares indicate
5 peptides showing significant abundance changes.
Representative results from two identified,
differentially expressed peptides are shown.

          Throughout this application various
10 publications have been referenced. The disclosures of
these publications in their entireties are hereby
incorporated by reference in this application in order
to more fully describe the state of the art to which
this invention pertains. Although the invention has
15 been described with reference to the examples provided
above, it should be understood that various
modifications can be made without departing from the
spirit of the invention.

50

What is claimed is:

1. A method for identifying and quantifying polypeptides in a sample, comprising the steps of:

5

(a) labeling peptides in a polypeptide sample with an isotope tag;

(b) adding a plurality of peptide standards
10 to said polypeptide sample, wherein said peptide standards are labeled with an isotopically distinct version of said isotope tag;

(c) resolving said labeled sample and
15 standard peptides into a plurality of fractions;

(d) analyzing said resolved fractions using mass spectrometry;

20 (e) identifying an isotope-tagged sample peptide in an analyzed fraction; and

(f) determining the amount of the identified isotope-tagged sample peptide in said analyzed fraction
25 by comparison to the amount of isotope tagged standard peptide in the same fraction.

2. The method of claim 1, wherein said plurality of fractions is deposited onto a mass
30 spectrometry sample plate.

3. The method of claim 1, wherein a known absolute amount of each of said peptide standards is added to said polypeptide sample.

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4.   The method of claim 1, wherein said
polypeptide sample is cleaved with a protease.

5.   The method of claim 4, wherein said
protease is trypsin.

6.   The method of claim 1, wherein said
sample is derived from a body fluid selected from the
group consisting of blood, plasma, cerebrospinal fluid,
urine, saliva, seminal plasma, and pancreatic juice.

7.   The method of claim 6, wherein said
sample is derived from serum.

8.   A method for quantifying polypeptides in
a sample, comprising the steps of:

(a)   labeling peptides in a polypeptide
sample with an isotope tag;

(b)   adding a known absolute amount of a
plurality of peptide standards to said polypeptide
sample, wherein said peptide standards are labeled with
an isotopically distinct version of said isotope tag;

(c)   resolving said labeled sample and
standard peptides into a plurality of fractions;

(d)   analyzing said resolved fractions using
mass spectrometry;

(e)   identifying an isotope-tagged sample
peptide in an analyzed fraction; and

(f)   determining the amount of the identified
isotope-tagged sample peptide in said analyzed fraction

by comparison to the amount of isotope tagged standard
peptide in the same fraction.

        9.   The method of claim 8, wherein said
5 plurality of fractions is deposited onto a mass
spectrometry sample plate.

        10.   The method of claim 8, wherein said
   polypeptide sample is cleaved with a protease.
10

        11.   The method of claim 10, wherein said
protease is trypsin.

        12.   The method of claim 8, wherein said
15 sample is derived from a body fluid selected from the
group consisting of blood, plasma, cerebrospinal fluid,
urine, saliva, seminal plasma, and pancreatic juice.

        13.   The method of claim 12, wherein
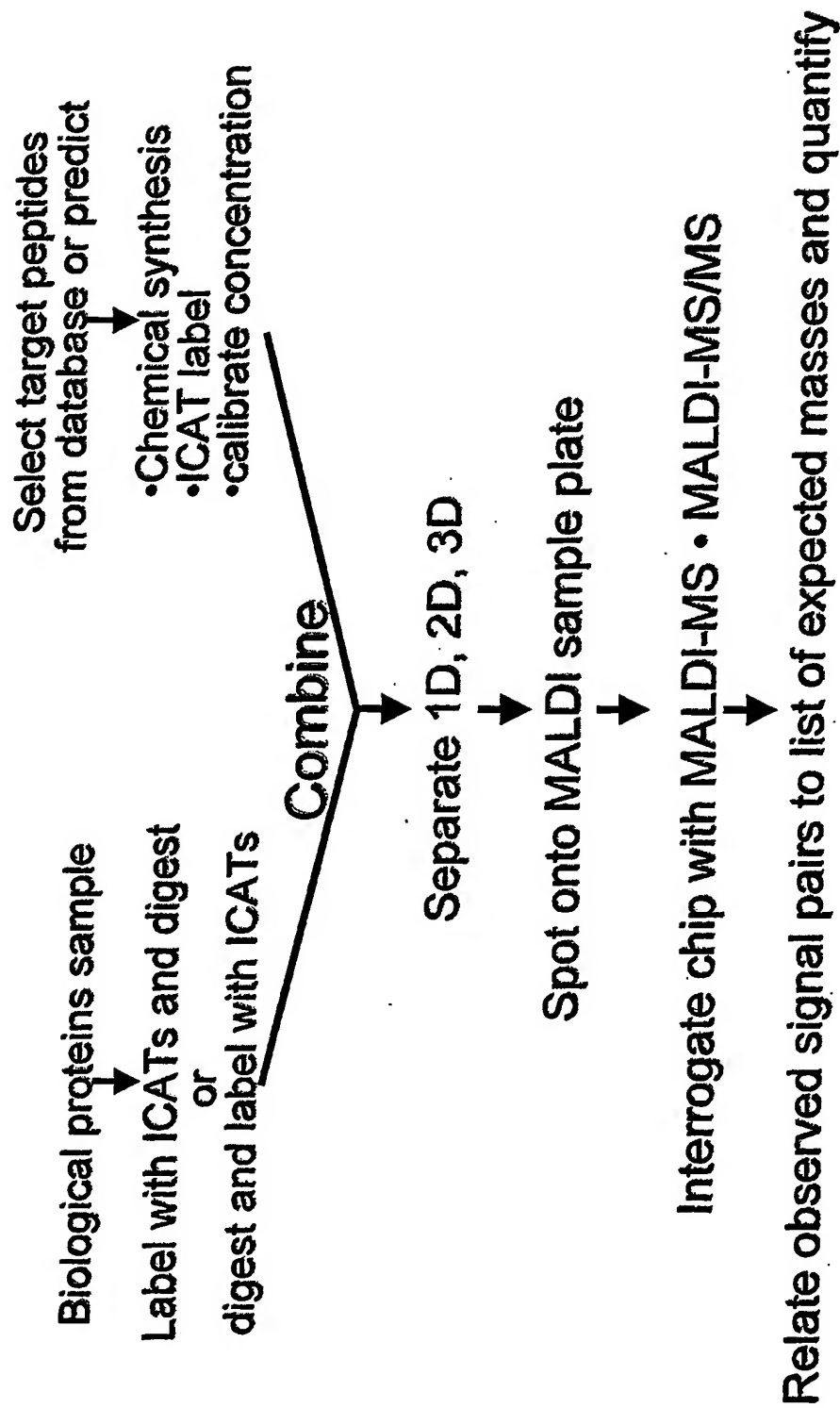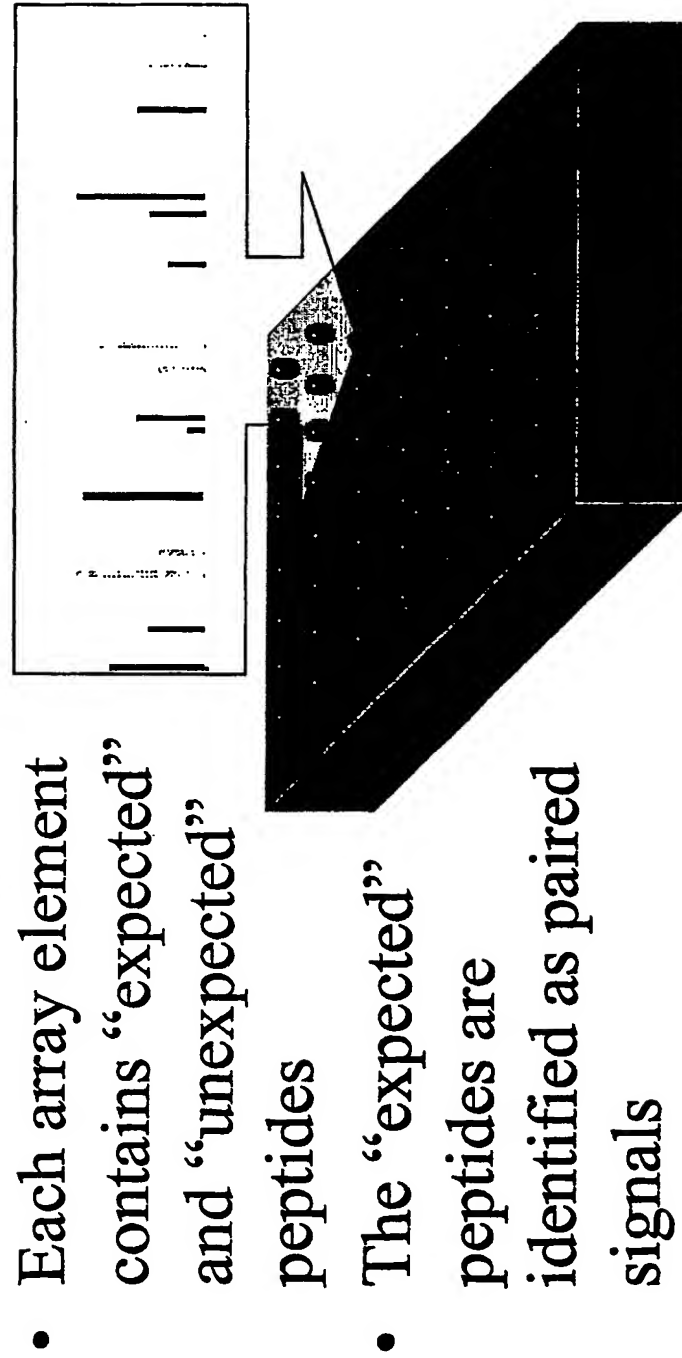20 saidsample is derived from serum.

        14.   A method for identifying and quantifying
   splice isoforms of polypeptides in a sample, comprising
   the steps of:
25

        (a)   labeling peptides in a polypeptide
sample with an isotope tag;

        (b)   adding a plurality of peptide standards
30 to said polypeptide sample, wherein said peptide
standards are labeled with an isotopically distinct
version of said isotope tag and wherein said plurality
of peptide standards comprises at least one peptide
corresponding to a common amino acid sequence of a
35 splice isoform of a polypeptide and at least one
peptide corresponding to an amino acid sequence that

differs between two splice isoforms of said
polypeptide;

(c)   resolving said labeled sample and
standard peptides into a plurality of fractions;

(d)   analyzing said resolved fractions using
mass spectrometry;

(e)   identifying an isotope-tagged sample
peptide in an analyzed fraction; and

(f)   determining the amount of the identified
isotope-tagged sample peptide in said analyzed fraction
by comparison to the amount of isotope tagged standard
peptide in the same fraction.

15.   The method of claim 14, wherein said
plurality of fractions is deposited onto a mass
spectrometry sample plate.

16.   The method of claim 14, wherein a known
absolute amount of each of said peptide standards is
added to said polypeptide sample.

17.   The method of claim 14, wherein said
polypeptide sample is cleaved with a protease.

18.   The method of claim 17, wherein said
protease is trypsin.

19.   The method of claim 14, wherein said
sample is derived from a body fluid selected from the
group consisting of blood, plasma, cerebrospinal fluid,
urine, saliva, seminal plasma, and pancreatic juice.

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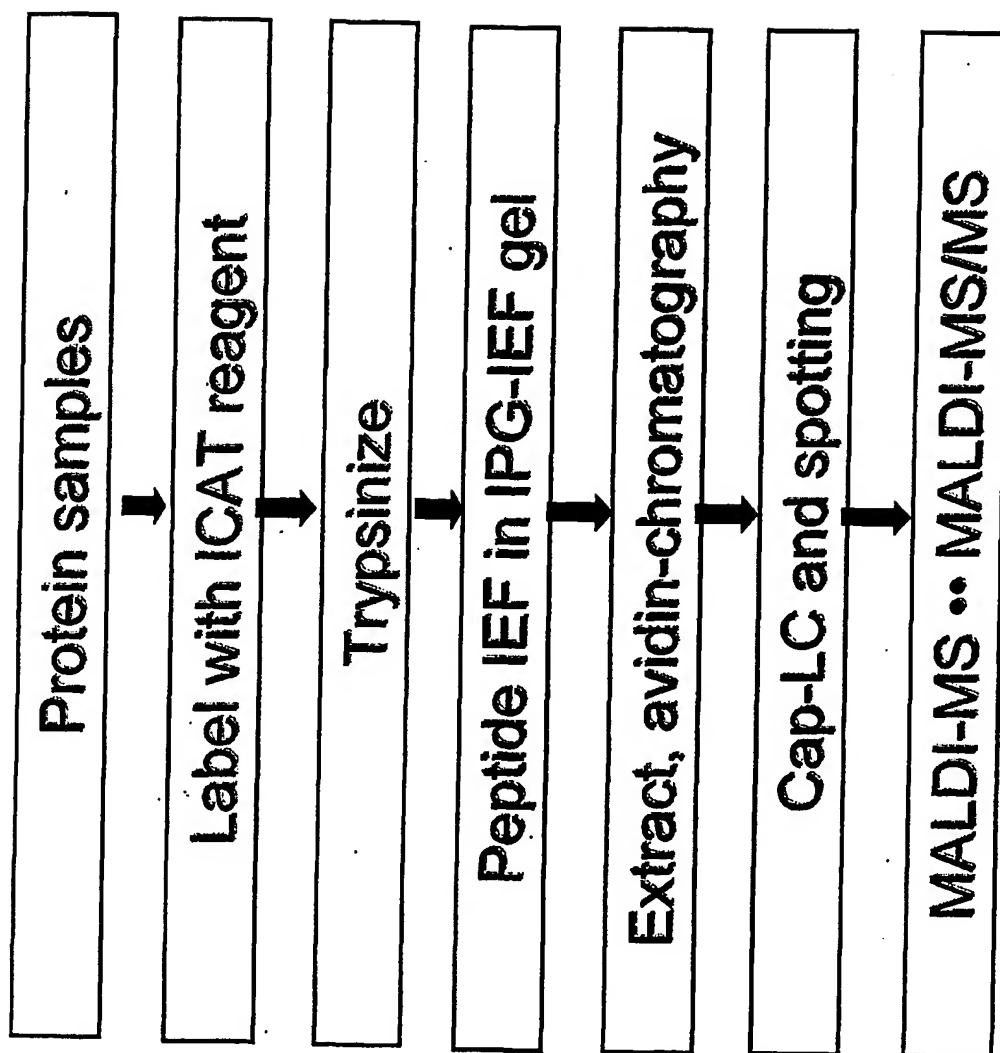20.　The method of claim 19, wherein said sample is derived from serum.

Select target peptides
from database or predict

•Chemical synthesis
•ICAT label
•calibrate concentration

Biological proteins sample

Label with ICATs and digest
or
digest and label with ICATs

Combine

Separate 1D, 2D, 3D

Spot onto MALDI sample plate

Interrogate chip with MALDI-MS • MALDI-MS/MS

Relate observed signal pairs to list of expected masses and quantify

FIGURE 1

FIGURE 2

- Each array element contains "expected" and "unexpected" peptides
- The "expected" peptides are identified as paired signals

FIGURE 3

Protein samples → Label with ICAT reagent → Trypsinize → Peptide IEF in IPG-IEF gel → Extract, avidin-chromatography → Cap-LC and spotting → MALDI-MS •• MALDI-MS/MS

FIGURE 4

- Dial in list of proteins (organelles, complex, splice forms, modifications, etc.,) to be profiled

- Robotic system selects suitable peptides from library of calibrated standards

Sample Prep

Separation

M S

Data Analysis

Anticipated throughput of 200 Hz MALDI-MS

One 96 well plate/min.

FIGURE 5

FIGURE 6

FIGURE 7

FIGURE 8

FIGURE 9